A03

(12) PATENT
(19) AUSTRALIAN PATENT OFFICE

(11) Application No. AU 199892366 B1
(10) Patent No. 709385

(54) Title
DNA fragment, recombinant DNA, and transformed plant

(51)[6] International Patent Classification(s)
C12N 015/29 A01H 004/00

(21) Application No: 199892366

(22) Application Date: 1998.11.12

(30) Priority Data

(31) Number	(32) Date	(33) Country
10-162186	1998.06.10	JP

(43) Publication Date : 1999.08.26
(43) Publication Journal Date : 1999.08.26
(44) Accepted Journal Date : 1999.08.26

(71) Applicant(s)
Kyushu University

(72) Inventor(s)
Koh Iba; Takiko Shimada; Tomonobu Kusano

(74) Agent/Attorney
GRIFFITH HACK,GPO Box 1285K,MELBOURNE VIC 3001

(56) Related Art
KUSANO T ET AL. (1995) MOL. GEN. GENET. 248:507-517

ABSTRACT

This invention relates to a DNA fragment comprising a base sequence (a) the base sequence referred to as nucleotide numbers 1-3794 in a sequence number 1 in a sequence list; (b) the base sequence (a) a part of which is deleted or substituted by another base sequence, or to which another base sequence is added. Further, the invention relates to a recombinant DNA and transformed plant including the above fragment. Moreover, the invention relates to fragment developing a promoter activity with a responsive property to low temperatures which comprises a base sequence (c) the base sequence referred to as nucleotide numbers 1-2797 in a sequence number 1 in a sequence list; (d) A part of the base sequence (c) developing a promoter activity with a responsive property to low temperatures; or (e) The base sequence (c) or (d) a part of which is deleted or substituted by another base sequence, or to which another base sequence is added. Further, the invention relates to a recombinant DNA and transformed plant including the above fragment.

AUSTRALIA

Patents Act 1990

COMPLETE SPECIFICATION

STANDARD PATENT

Applicant(s):

KYUSHU UNIVERSITY

Invention Title:

DNA FRAGMENT, RECOMBINANT DNA, AND TRANSFORMED PLANT

The following statement is a full description of this invention, including the best method of performing it known to me/us:

DNA FRAGMENT, RECOMBINANT DNA, AND TRANSFORMED PLANT

BACKGROUND OF THE INVENTION

[0001]

1. Field of the Invention

This invention relates to a DNA fragment, a recombinant DNA suitable to produce a breed such as a maize or rice having a low temperature resistance, and a transformed plant.

[0002]

2. Description of Related Art

From so far studies, it is found that there is a significant relationship between a low temperature resistance of a plant and a degree of unsaturation of fatty acid constructing a biomembrane thereof.

SUMMARY OF THE INVENTION

The inventors experimentally indicated that a transformed tobacco plant comes to have a higher resistance against low temperatures when a fatty acid unsaturating enzyme gene FAD7 derived from an arabidopsis is highly expressed in the plant.

[0003]

When a production of a certain protein in a plant cell is required, a promoter operating constitutively and having a high promoter activity has been used. Such a promoter operates continuously, which is futile in the plant.

[0004]

For instance, the constitutive promoter is also employed when a protein giving a plant a low temperature

resistance is phenotypically expressed. Therefore, even at ordinary temperatures, the protein for giving the plant the low temperature resistance is forced to be unnecessarily expressed, causing unfavorable results.

For such reasons, a development of a site-specific and inducible promoter has been required. Particularly, it is required that an expression of a specific gene is strengthened only at low temperatures, in order to produce a breeding intermediate mother body in which a gene expressing an enzyme unsaturating a fatty acid and other protein genes contributing to a low temperature resistance is inducibly expressed in response to low temperatures, or in order to make possible production of an unstable functional protein using a plant cell.

The invention provides a promoter capable of strengthening expression of a fatty-acid-unsaturating-enzyme gene and other protein genes contributing to a low temperature resistance in response to low temperatures.

The invention further provides a breed having a low temperature resistance using the above promoter.

The invention also provides a means of utilizing the promoter in an inducibly generating system of a functional protein in a plant cell being specific to low temperatures.

According to an aspect of the invention, there is the provision of a recombinant DNA and transformed plant having a low temperature resistance and induced a recombinant DNA including the DNA fragment mentioned above, respectively.

According to a second aspect of the invention, there is the provision of a recombinant DNA which includes the DNA fragment comprising the base sequence (c), (d), or (e) described above, and a transformed plant which transduces the recombinant DNA and phenotypically expresses a specific protein in response to low temperatures.

Since a rice's low temperature inducible gene lip19 or maize's low temperature inducible gene mlip15

codes a DNA binding factor, they are considered to be a gene controlling transcription of other gene groups induced or repressed under low temperature stress. The inventors have clarified a functional unit of a low temperature inducible promoter in the maize's mlip15 gene and that the functional unit also operates in a transformed plant.

The inventors has isolated a genomic clone of the maize's mlip15 gene by an common method. Due to determination of nucleotide sequence, it becomes clear that the gene does not include a intron (see: Sequence number 1 in Sequence list).

In the gene mlip15, 2.8 kb of a nucleotide sequence (0.6 kb of a nontranslated region at 5'-end and 2.2 kb of a nucleotide sequence linking at upstream thereof, in a mlip15 cDNA) and 2.2 kb of a nucleotide sequence (the nucleotide sequence remained after subtracting 0.6 kb of the non-translated region at 5'-end from 2.8 kb of the nucleotide sequence) are respectively bound with a β-glucuronidase gene as a reporter gene to make recombinant DNAs.

Each recombinant DNA is introduced into a callus derived from a rice's scutellum by using a particle gun.

PAGES 4 and 5 ARE INTENTIONALLY BLANK

The result is that the former maintains a reactivity for low temperatures but the latter lose it. Therefore, it has been clarified that the 2.8 kb fragment containing 0.6 kb of the nontranslated region at 5'-end has a promoter function responsive to low temperatures.

[0017]

In the invention, as a vector used for producing a recombinant DNA, a plasmid can, for example, be employed. Further, as a plant used for introducing the recombinant DNA, a useful cultivating monocotyledon such as maize, rice, wheat, barley, oat, Italian millet, or Japanese millet is preferably used.

A protein produced by induction of the promoter according to the invention includes an ω-3-fatty acid unsaturating enzyme etc.

Moreover, the invention includes a base sequence one or several nucleotides of which are deleted or substituted by another base sequence, or to which another base sequence is added, if it maintains the promoter function according to the invention.

BRIEF DESCRIPTION OF THE DRAWING

The invention will be described with reference to the accompanying drawing, wherein:

Fig. 1 is an illustration diagrammatically showing mlip15 region.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0018]

(Isolation of mlip15 genomic clone and determination of the

nucleotide sequence)

A genome DNA prepared from a maize (breed: honey bantam) is cut (partially digested) by a restriction enzyme Sau3A and then separated by using a sucrose density gradient centrifugation to obtain 9.7-22 kb of DNA fraction. The DNA fraction is bound with a λ-EMBL3 digested by BamHI and then makes phage particles by using Giga·packGoldIIkit.

By examinating them by using Escherichia coli XL1-Blue MRA (P2) as a host, $1X10^7$ pfu/ml of a library is obtained.

The library is selected by using the whole length of the mlip15 cDNA as a probe to obtain three positive clones.

Among them, a clone named "λH1" is found to include 11.5 kb of a fragment containing the mlip15 cDNA, 5 kb of a fragment at the 5'-end, upstream thereof, and 5 kb of fragment at the 3'-end, downstream thereof.

[0019]

In Sequence list 1 is shown 3,794 bp of a base sequence being an EcoRI-BamHI fragment including (consisting of) the whole length of the mlip15 cDNA.

A putative amino acid sequence at the region coding an mlip15 protein is symbolized under the base sequence by one capital letter. Nucleotide numbers corresponding to the assumed amino acid sequence are 2798-3205. A terminal codon of the base sequence is shown as a mark "*". A nucleotide sequence which is assumed as "TATA box" is underlined and also has a description of "TATA box" thereunder. A transcription initiation point of mlip15 is T as nucleotide number 2272.

[0020]

(Constructing of recombinant DNA used for introducing to rice'callus)

A fragment having the nucleotide numbers 1-2797 and a fragment having the nucleotide numbers 1-2271 are respectively amplified by a PCR method (a polymerase chain reaction method) using the maize'mlip15 genomic clone as a template. The fragment of the nucleotide numbers 1-2797 is the above mentioned genomic sequence of 2.8 kb which consists of 0.6 kb of the nontranslated region at 5'-end and 2.2 kb of genomic sequence at upstream of the 0.6 kb of the non-translated region at 5'-end, in mlip15 cDNA . The fragment of the nucleotide numbers 1-2271 is the above mentioned genomic sequence of 2.2 kb and consists of the rest of 2.8 kb of the nucleotide sequence subtracted by 0.6 kb of the nontranslated region at 5'-end.

A nucleotide numbers 2798-3205 is a region coding the mlip15 protein.

[0021]

As an enzyme in the above amplification, LATaqDNA polymerase having a proofreading activity is used. In this case, each primer is such designed that the base sequence of the nucleotide numbers 1-6 becomes a Hind III site (AAGCTT) and the base sequence of the nucleotide numbers 2792-2797 and the base sequence of the nucleotide numbers 2266-2271 respectively become a Bam HI site (GGATCC) to obtain each primer.

[0022]

Each amplified fragment is integrated between the

Bam HI and Hind III sites of a vector used for an Escherichia coli pUC18 and a confirmation of each base sequence is conducted. Using each plasmid with each base sequence confirmed, each Bam HI - Hind III fragment respectively consisting of 2797 bp and 2271 bp is reproduced and integrated between the Bam HI and Hind III sites of pBI 221. Each obtained recombinant plasmid is respectively named pBImp28 (because of containing 2.8 kb of promoter region in the mlip15) and pBImp22 (because of containing 2.2 kb of promoter region in the mlip15).

[0023]

In Figure 1, the mlip15 promoter region is diagrammatically illustrated. Beside, the mlip15 promoter portion contained in the above recombinant plasmid is shown therein. The symbol of "+1" indicates a transcription initiation point (the nucleotide number 2272), the symbol of "key arrow" the direction of transcription, and the symbol of "ATG" the position of a transcription initiation codon (the nucleotide number 2798). A β-glucuronidase gene (abbreviated as GUS) is used as a reporter gene (its scale is not correct in the figure).

[0024]

(Reactivity of the introduced plasmid in low temperatures)

To a callus derived from a rice' scutellum (breed: Notohikari), the above each plasmid is introduced using a particle-gun (made by BIO-RAD Co., Ltd.) After the introduction, the callus is incubated for 24 hours in a dark place at 25°C and then divided to two parts homogeneously. After

respectively incubating for another 24 hours one at 25°C and the other at 5°C, a GUS test is carried therefor. A GUS activity is judged as whether a low temperature reactivity is present or not, by calculating a ratio of the number of blue spots per scutellum on an X-Gluc as substrate at 5°C to that of 25°C (see: Table 1). Three times of tests are repeated on both plasmids and the results are shown in Table 1.

[0025]

Table 1

Induced plasmid	Responsive property to low temperatures Ratio of GUS activity(5°C/25°C)	
pBImp28	Experimental 1	2.7
	Experimental 2	6.0
	Experimental 3	4.7
pBImp22	Experimental 1	1.1
	Experimental 2	0.62
	Experimental 3	0.57

[0026]

When the pBImp28 is introduced, a high GUS activity is obtained. But, when the pBImp22 is introduced, the GUS activity is inferior thereto. The following may be derived from these results.

(a) In DNA fragment of the nucleotide numbers 1-3794 of the maize' mlip15 gemonic clone, the promoter region relating to the low temperature responsive property exists at upstream of the putative amino acid sequence coding the mlip15 protein.

(b) The fragment of the nucleotide numbers 1-2797 contains the promoter region relating to the low temperature

responsive property.

(c) The fragment of the nucleotide numbers 1-2271 is expected to effect as the promoter, but the fragment itself has a low responsive property to low temperatures. The fragment itself of the nucleotide numbers 2272-2797 has the low temperature responsive property and relates to the expression of the promoter.

[0027]

Sequence list

Applicant name : President of Kyushu university

Title of invention : DNA fragment, recombinant DNA, and transformed plant

Number of sequence : 1

Sequence number : 1

Sequence length : 3794

Sequence type : Nucleic acid

Number of strand : Double strands

Topology : Linear

Original source

Organism : Maize breed of honey bantam

Genome of mlip15 gene

Sequence: Genomic sequence of mlip15

```
   1 GAATTCCGAATAACGCGCCCCGCATGCAACCAGATAGCGGATCTTTCGGCGCTAAACTCA   60
  61 GAGGGAAGCAATTGCCGGAAGAGTCGGCGTGCAAGAATAACATAAGTAGATAAGATTTCA  120
 121 CGATCTATAAAAGGATATCTCCCTAGTCGGCTATATAAGGCTAGGGAGGTACCCAAACAA  180
 181 AACGAATCACTCTCTTTCACCACCATAACGCCCACTAGTAGACTAATATGAGATCTCATC  240
 241 CACCGTCACCCGCGAATCATCTGTAACCCAAGCAAACTCAATACCCAACATCACACATGA  300
 301 CTTAGGGTATTACGCATTTAGGCGACCCGAACCTGTATAATTTTCTTGTGTTTCAACGTG  360
```

361 CACCTGCACGTACCATCGAGTTGCGATTAACGTCGCCGTCCTCCCAAAAATACTGGTGGT 420
421 CCTCCCAAAAACTCGACCGGACGATAGAAATAGTGTATGGCTAAGTAGAGCAAGGTGGCG 480
481 TTTGGTCCGCGGCTATGATGAGAATCCAGTAGACTGAATCCACGTGCTAAACCAAACATC 540
541 ACTGGTTTTGGCTGCATCTTCTCGGATTACGTGTGTTGTGCAAATTCTCATAATCCACGG 600
601 CAACCAGACGGGGCGCAACCAATTGGTGTTTCTTAGGAAGCCCCCGCGTTACATTGGAT 660
661 CATAGGATGATTCAAGGGTTATGATTTTTTAGCTACTAATTGGTTGTCATCATGGTTTAT 720
721 AGGTGAAGATTGTTATTCAATCAAAGGGCGACATATCCCTCCGCGTTTAGAGACTTGCGT 780
781 GTAGTGTAAACATGGATGTAATTGTGCTACCTTTAATAGAGTCCCTTAGCTCTTCAAAAA 840
841 CAAATCTTATTATATATTAATTACTAGTCCATCCATTTTATTCTAATTTAGTTTCGAAAT 900
901 TACTAAATATAGAAAATAAAATAGAGTTTTAGTAGCAATTATGAAAACTGAAATATAGTT 960
961 TTAATTTCCGTATTTAGTGATTTAAATACTAAAATATAATAAAATGGAGAGACTAAAAAC 1020
1021 TAGTCCCTATAAACCAAACATTCTTTAAATAAAGCCCCGTGGCTAGGACAATGACCTATT 1080
1081 TTTTTCTCGCAACCGGAAGAATAAAAAATTCACCGTAACTTTCTTTCTTTCTTCTTTTTA 1140
1141 TGCGAAAGAAGATAGTTGCAAGACGAATCCAGAGTTTATCTGGAAGAAGAAAGTTCCTAA 1200
1201 TCCTCCTCCTTCCCTGTAGATATTATCAGCAAGGCAAGCGTGTCACGGCTTCTTGCTTGA 1260
1261 GTAATCCGCTCCTATTTTTTTTTTTGGGAGGGCGCCTTCCTACCGCCTTCGCTTCTAAAC 1320
1321 GGTGGGCAAATTTGGTACGATAAAGAAAAAAGAGGAGGACGAGTGGGAGGGCACTTCTGG 1380
1381 AAAAAACTTTTTAATGAGCTGGACCAAGCAGCTGGGCAAGCTGTCACTAGGACTGGACAA 1440
1441 AATACTCGTGGCTCGATAACTCGCTCGACTCGGCTCGTTAGTAGCTCAGCTCGACTCGGC 1500
1501 TCGTTTTAATTTTGTAGCGAGCCAAGCTAGCATTCTAGCTCGATTCTCTAATGAGCCAGC 1560
1561 TCGGGTTAGCTCGTGAGCTAGCTCGCGAGCCAAACGAGCTAAGCCACAACACAAATTTGT 1620
1621 CTAGTCATTGATGTCGTCTCATCTCTCATAGTCTTGTTTTCTCGTAGTTATGATCTGTGA 1680
1681 TATGGACATGTGTGGATGTGCCATGTGCTTAAATATTTATATTATTGCATGGCTACATGT 1740
1741 TTGTAGTGTTAAATACTTAAAATATAATTTTTCGGTTATAAATATATTTATGTACATAGA 1800
1801 TATTTATATTTAGTTGTGTGGCTCACGAGCCTAACGAGCTGGCTCGAGCTTCCTAACGAG 1860
1861 CCGAGCCGAGCCAGCTATTTAGCTCGTTAGTATAACGAGCCGAGCCGAGCTGGCTCGTTA 1920
1921 TAGTAACGAGCCATAACGAGCCGAGCCATAACGAGCCAAGCTGGTTCGATATCCACCCCT 1980
1981 AGCTGTCACCGTCGCCCAGTCCGCTTCGTTCGGTCAGCGGGCCCCACCTCATCTGCATTC 2040

```
2041 TTCCATTCTCGTCCTCCGACCTCATCTGCATTTTCCCAGCCAAGTAGTAGGTAAACTAGT 2100
2101 GGCGGTCCCGTGGCCGTGGCATCAGGAAAAGAATATGCCGTCCCAGCCCACCATCCCCCC 2160
2161 ACCGTCCCGAAATTCCAGAACTACCCTCGGCTCCAGCTATAAATAGCCGCCCCCGGGAGA 2220
                                          putative TATA box
2221 CGTTCGAAACCTTCCCCATCTCCGGATAAAAGATAAGGAGTGTCTCTCCTCTCTTTCAGC 2280
                                                        cDNA start site
2281 TAAGTCCCTGCTCCCTCTCTTTTTCTTACATTCAGGTCCTCGCAGCTCCTCTCTTTTTTC 2340
2341 TTGTTTCTTTCTTTCGATCTGCGAGCCGTCCAGGTCCAGTACTCTCCTTTCCGTGAAGGA 2400
2401 ACTCTTGCAGCCGGCCCCTCTGGTTTCCTCGAATTCTTGTTCCCCGGTCCCTCCTCCTGT 2460
2461 CCCCGCGTAGATCCGTCCGTCCGAGGAGCACACCGTCCCCACCCCCATGTTTACCCACCA 2520
2521 GTTCCTCTGACGGCCGCCGTGCTCCGATGAAGCTGAGCGTGCTCCGTATCCGCCGCTCCC 2580
2581 ACTCCTTCTCCGTCGCCTTCCTCTACTGGTTCTACGTCTTCTCATGAACGCATCGCCCCT 2640
2641 CTCCACCTGCTGATCCTTCGCCATCTCTCCATCTCTCTTTCTCTCTGAGATAGTCTTTCG 2700
2701 AATCCATCTCTAGGGCTCTTGTTTCTCCCCATCCTCCCCCCACCCCACCCCCCCACCAAAC 2760
2761 ACAAGTCCCCTTGTTCAATCCGACAAGACAAGCATCCATGTCGTCGTCACGCCGGAGCTC 2820
                                                  M  S  S  S  R  R  S  S
2821 GAGCCCCGACAGCAACGACACGACGGACGAGCGCAAGCGGAAGCGGATGCTGTCCAACAG 2880
     S  P  D  S  N  D  T  T  D  E  R  K  R  K  R  M  L  S  N  R
2881 GGAGTCGGCGCGGCGGTCGCGCGCGCGGAAGCAGCAGCGGCTGGAGGAGCTGGTGGCGGA 2940
     E  S  A  R  R  S  R  A  R  K  Q  Q  R  L  E  E  L  V  A  E
2941 GGTGGCCCGCCTGCAGGCGGAGAACGCGGCGACGCAGGCCCGCACCGCGGCGCTGGAGCG 3000
     V  A  R  L  Q  A  E  N  A  A  T  Q  A  R  T  A  A  L  E  R
3001 CGACCTGGGCAGGGTGGACGGCGACAACGCGGTCGTGCGCGCCCGCCACGCCGAGCTGGC 3060
     D  L  G  R  V  D  G  D  N  A  V  V  R  A  R  H  A  E  L  A
3061 CGGCCGCCTGCAGTCGCTGGGCGGCGTCCTCGAGGTGCTCCAGATGGCCGGCGCCGCCGT 3120
     G  R  L  Q  S  L  G  G  V  L  E  V  L  Q  M  A  G  A  A  V
3121 CGACATCCCGGAGATGGTCACCGACGACCCCATGCTCCGCCCCTGGCAGCCGTCCTTCCC 3180
     D  I  P  E  M  V  T  D  D  P  M  L  R  P  W  Q  P  S  F  P
```

```
3181 CCCGATGCAGCCCATCGGGTTCTGAGAATCTGAGCCTCAGCCGGCGGGAGAGAGCCAATT 3240
     P  M  Q  P  I  G  F  *
3241 TCTGTCGTCGTGCCGCTGTCTATCTCGTATTGGTATATCTATTCATAAATCATCCTTGTC 3300
3301 ATGGTTTGCTCTTCTTGTTCAGTGTTATAAATTTGCTTCTTGTTAGTGTTATAAATTTGG 3360
3361 CCATCGGAAAGGATGTGTTTGTAGTTGTAATATCTTGTTTGGAGTTGTAATATCTTATCT 3420
3421 TGCTTATGAAATCGAATATGCCTATATATATATGTTATGCTGTACGAGTATGTGGCTCCA 3480
                                   polyA additional site
3481 AATTTGTGAGCCTTCTGTCTGTTATGGTGAGGCGATGAATCCAATTTGTGAGCACACATG 3540
3541 AATCAATTTCGAGATTCGACATGTCAAGTTGATCGTTGCAGGAAGGACGGTTTTTGTATG 3600
3601 GACGGACATACCAAGTTACTGCATTTTACTTAAAATATCTCACTTATTTTTTAGATCGGC 3660
3661 ATTTCTCCACTCGTTAGATTTCTTGTTCTTGAGTCAGAAGATAACTACAGCATGTCATAT 3720
3721 CTCAATTGGAATACCATTAGGGTCCCTCATCTTAACCTATTTCATCTCTTTTAATACGTA 3780
3781 GATTTTTTGGATCC 3794
```

In the claims which follow and in the preceding description of the invention, except where the context requires otherwise due to express language or necessary implication, the word "comprising" is used in the sense of "including", i.e. the features specified may be associated with further features in various embodiments of the invention.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A DNA molecule comprising the nucleotide sequence 1 to 3794 shown in sequence 1, or a functional fragment thereof, wherein said DNA codes for a promoter which is induced by low temperature or a structural gene coding for mlip15 protein.

2. A DNA molecule comprising the nucleotide sequence 1 to 2797 shown in sequence 1, or a functional fragment thereof, wherein said DNA codes for a promoter which is induced by low temperature.

3. A DNA molecule comprising the nucleotide sequence 1 to 2271 shown in sequence 1, or a functional fragment thereof, wherein said DNA codes for a promoter which is induced by low temperature.

4. A DNA molecule which has at least 75% sequence homology with a DNA according to any one of claims 1 to 3.

5. A DNA molecule which has at least 85% sequence homology with a DNA according to any one of claims 1 to 3.

6. A DNA molecule which has at least 95% sequence homology with a DNA according to any one of claims 1 to 3.

7. A recombinant DNA molecule comprising a DNA molecule according to any one of claims 1 to 6.

8. A transformed plant having a low tempearture resistance, wherein said plant comprises a DNA molecule according to any one of claims 1 to 7.

9. A transformed plant phenotypically expressing a specific protein in response to low temperature. wherein said plant comprises a DNA molecule according to any one of claims 1 to 7.

10. A DNA according to claim 1 substantially as hereinbefore described with reference to any of the examples.

Dated this 25th day of June 1999

KYUSHU UNIVERSITY

By their Patent Attorneys

GRIFFITH HACK

Fellows Institute of Patent and

Trade Mark Attorneys of Australia

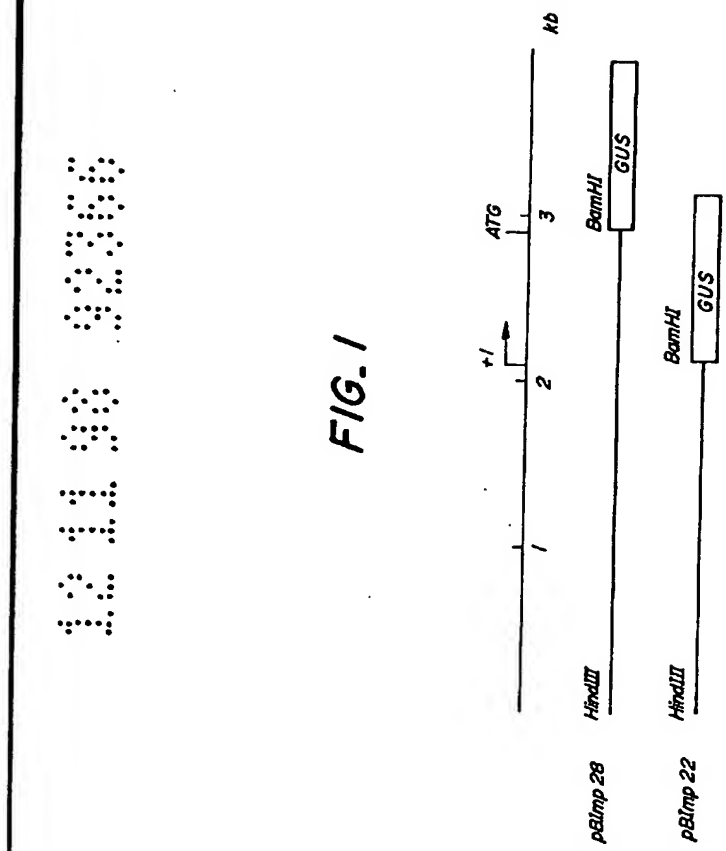
kb
ATG
3
+1
2
1
BamHI
GUS
HindIII
pBImp 28
BamHI
GUS
HindIII
pBImp 22

FIG. 1